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The Missing Link between *Candida albicans* Hyphal Morphogenesis and Host Cell Damage

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Introduction

Fungal pathogens are more commonly associated with morbidity and mortality than generally appreciated. In fact, a significant portion of the world population is infected by fungi, and an estimated 1.5 million people die from life-threatening fungal infections each year [1]. One of the most common fungal pathogens of humans is *Candida albicans*. The majority of the human population is colonised with this fungus, and superficial infections of mucosal surfaces are extremely common [2].

The morphological plasticity of *C. albicans* has long been implicated in the virulence of this pathogen [3]. The two most important morphologies, yeast and hyphal cells, are both required for virulence. Neither yeast-locked strains nor hyperfilamentous mutants are fully virulent in experimental systemic infections. However, it is generally accepted that each of the two forms fulfils specific functions during infection. While the yeast form is likely important for dissemination via the blood stream, the formation of filamentous hyphae contributes to adhesion and invasion of host cells.

What's Special about Hyphae?

The invasive nature of hyphae is intuitive and supported by multiple studies (Fig 1). (i) Hyphae are the most common morphology observed during experimental infections and in patient biopsies, and histological analysis clearly shows that hyphae are the dominant invasive form [4]. (ii) Hyphae adhere more robustly and efficiently to host cells than yeast cells, largely owing to two hypha-associated adhesins, Als3 and Hwp1 [5] (Fig 1a). However, in certain environments, such as dynamic endothelial-interactions, yeast cells [6] or short germ tubes [7] have been reported to be more adherent than longer hyphae. (iii) Only hyphae invade efficiently into human cells, which occurs via two routes; induced endocytosis and active penetration [8] (Fig 1b). Induced endocytosis is mediated by the hypha-associated invasin, Als3, and is mainly dependent on host activities—even killed hyphae are endocytosed as long as Als3 is expressed on their surface. Active penetration, on the other hand, is a fungal-driven process that requires fungal viability but not host activity. Both invasion routes require hyphae, and mutants defective in hypha formation are also defective in host cell invasion [9]. However, hypha-mediated invasion of host cells by either route does not necessarily cause cell damage (Fig 1a–1c). Whilst *C. albicans* hyphae formation appears to play a central role in host tissue invasion, other morphotypes are critical during infections of other host niches. For example, yeast cell dispersal

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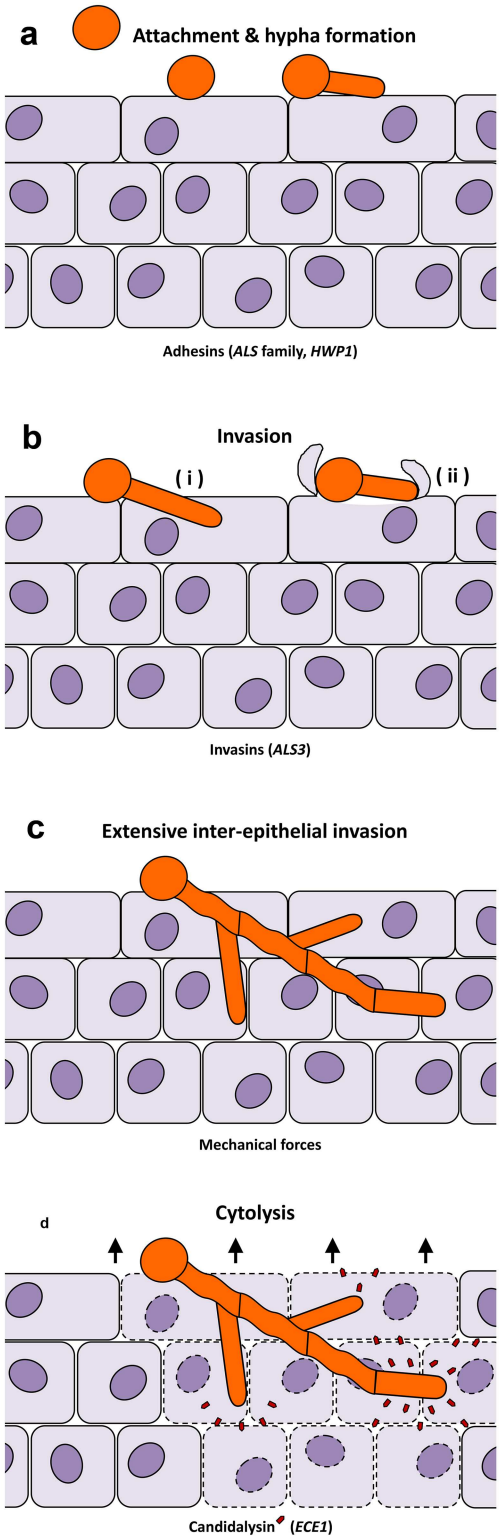

Fig 1. Distinct stages of *C. albicans*-epithelial infection. (**a**) In experimental epithelial infections, *C. albicans* yeasts form hyphae upon contact with epithelia and adhere tightly to the host cells. This is mediated by a number of adhesins, including members of the Als family and Hwp1. (**b**) This is followed by initial epithelial invasion via two routes—(i) fungal-driven active penetration and (ii) host-mediated induced endocytosis. (**c**) Elongating and branching hyphae result in extensive interepithelial invasion. Surprisingly,

this invasion itself does not cause damage to the epithelium. (**d**) Simultaneous secretion of the fungal peptide toxin, Candidalysin (red pentagons), lyses the host epithelia and causes tissue destruction.

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likely plays a key role in seeding the bloodstream from biofilms formed on indwelling medical devices [10]. (iv) Hyphal cells are involved in trace metal acquisition. During the transition from commensalism to invasion, *C. albicans* utilises different assimilation strategies to gain nutrients from host cells. Hyphae of *C. albicans* can efficiently bind the host iron storage protein ferritin [11] and host zinc [12] during invasion of epithelial or endothelial cells, promoting fungal growth. Notably, the *C. albicans* ferritin-binding protein is Als3, suggesting multiple virulence functions for this protein, including adhesion, invasion, and iron acquisition. The pH-regulated antigen 1 (Pra1) acts as a secreted zinc-binding protein and also possesses immune evasion functions via binding complement regulators and thereby avoiding complement deposition [13]. (v) Hyphae facilitate fungal escape from phagocytes and induce macrophage killing via a two-step mechanism: initiation of pyroptosis and piercing of the macrophage membrane [14]. (vi) Finally, the expression of other virulence-associated genes is linked to the morphological transition. These include hypha-associated secreted aspartyl protease genes (*SAP4-6*) [15] and the superoxide dismutase gene *SOD5* [16], but also a small set of eight core response genes, which are expressed under hypha-inducing conditions [17]. These hypha-associated virulence genes may have distinct functions for invasion processes and may prepare the invading fungal cells for impending host niches [18]. Therefore, hypha development is coupled to multiple invasion-associated properties, but if invasion per se does not directly damage host cells, how does this process occur?

How Do Hyphae Damage Host Tissue?

As discussed above, hypha formation has long been known to be associated with a number of pathogenic properties and is a prerequisite for damage induction. However, the identification of a specific *C. albicans* factor that directly induces cell damage had remained elusive. This missing link between hyphal morphogenesis and damage induction has now been identified as a cytolytic toxin called Candidalysin, a 31 amino acid peptide [19] (Fig 1d). Candidalysin is generated from its parent protein, Ece1, which is encoded by the gene *ECE1*. *ECE1* is one of the eight core filamentation genes in *C. albicans* and was first discovered in the 1990s due to its high expression during hypha formation [20]. However, its molecular function remained unknown for almost a quarter of a century. In silico analysis suggested that Ece1 is a polypeptide consisting of a secretion signal peptide followed by eight short peptides, each separated by lysine/arginine residues. Previous studies had shown that these dibasic amino acids can be recognised by a subtilisin-like serine protease, Kex2, in the Golgi apparatus [21]. Proteomic analysis confirmed that Ece1 is produced by *C. albicans* hyphae and is sequentially processed at arginine/lysine residues by Kex2 and another serine protease, Kex1, respectively, followed by peptide secretion [19]. Candidalysin is one of these peptides. Candidalysin adopts an α-helical structure and, when secreted in sufficient quantities, intercalates and permeabilises host epithelial membranes to induce cell lysis. The presence of cholesterol in target membranes enhanced the lytic activity of Candidalysin, suggesting that membrane sterols may contribute to target specificity. Additional molecular analyses demonstrated the importance of Candidalysin, since deletion of only the Candidalysin-encoding region from the *ECE1* gene abolished the ability of *C. albicans* to damage epithelial cells in vitro and significantly attenuated *C. albicans* virulence in two in vivo models of mucosal infection: a cortisone acetate-treated mouse model of oropharyngeal candidiasis and a zebrafish swim bladder infection model [19]. Therefore, it appears

that production of Candidalysin rather than hypha formation per se is the mediator of host cell damage. Given that Candidalysin is a hypha-associated factor, these observations finally provide the elusive missing link between filamentation and host cell damage and explain why *C. albicans* hyphae are the destructive morphology during mucosal infections. This work also identifies Candidalysin as one of the very few "classical virulence factors" in human pathogenic fungi [22].

How Do Epithelial Cells Detect Candidalysin to Induce Immunity?

While Candidalysin is critical for fungal pathogenicity, our immune system is not helpless against this peptide toxin. Candidalysin is recognised by host epithelial cells and has been identified as the hyphal moiety that triggers the "danger response" pathway in epithelial cells. This pathway comprises NF-kB and PI3K signalling along with strong activation of MAPK signalling, resulting in activation of the transcription factor c-Fos via the p38 pathway and MKP1 via the ERK1/2 pathway [23,24]. Hence, when encountering yeast cells, our mucosal tissues tolerate these as benign colonisers but when encountering damage-inducing hyphae, Candidalysin induces the danger response pathway. In this way, the host is able to discriminate between the commensal and pathogenic states of *C. albicans*. These signalling events ultimately induce epithelial cytokine production and recruit immune cells (phagocytes and dendritic cells) to defend against infection. Intriguingly, the epithelial danger response has learned to respond to Candidalysin at levels below those required to induce cell lysis. For example, p-MKP1, c-Fos, and the nondamage-associated cytokine G-CSF were induced by sublytic concentrations ($\leq$3 μM) of Candidalysin, and by a modified nontoxic version of the peptide. We propose that this dual function of Candidalysin is the result of a coevolutionary event; the fungus has developed an efficient peptide toxin to damage host membranes and, in response, the host has evolved a sensitive Candidalysin detection system to identify and defend itself against this common mucosal pathogen.

Given the worldwide prevalence of mucosal *C. albicans* infections [1], the identification of the first cytolytic peptide toxin produced by a human fungal pathogen has therapeutic potential for the treatment of mucosal candidiasis.

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